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FIRST CLASS MAIL CERTIFICATE

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Date

Commissioner for Patents
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DECLARATION UNDER 37 CFR § 1.132

Sir:

I, James P. Fandl, declare as follows:

1. I am a co-inventor of the invention described and claimed in the above-identified patent application. I presently hold the position of Vice President at Regeneron Pharmaceuticals, Inc., having its principal place of business at 777 Old Saw Mill River Road, Tarrytown, New York 10591.
2. I am aware that the instant application claims our method of isolating cells based on the level of secreted protein expressed. This method, termed FASTR (FACS-dependent autologous secretion trap), requires the construction of a host cell line that expresses a cell surface capture molecule (CSCM) capable of binding a secreted protein of interest (sPOI).
3. Since the filing of the present application, I have performed additional experiments that provide further confirmation that any cell surface-bound protein can be a CSCM if it has some

measurable affinity for the sPOI. These additional experiments (Examples 1-4) are described below.

4. Example 1 investigated the effect of cell surface capture molecule (CSCM) expression level on the ability to identify high expressing cells through FASTR screening. Two host cell lines with different expression levels of the cell surface capture protein hFcγRI were analyzed. The high expressing RGC10 cell line contains 40 copies of the hFcγRI gene stably integrated via pTE158. A low expressing cell line, RS527, was generated from CHO K1 after stable transfection and selection for single copy gene integration. RS527 cells expressed significantly less hFcγRI protein than RGC10 cells as determined by Western blot analysis (Fig. 1).

RGC10 and RS527 cells were transfected with pTE462, a plasmid capable of expressing a hFc-fusion protein (Rc1-hFc) and conferring resistance to hygromycin. The transfected cultures were selected with hygromycin for two weeks. The hygromycin-resistant cells were induced with 1 μg/ml doxycycline (Dox) and blocked with rabbit IgG overnight. The next day, the RGC10/pTE462 and RS527/pTE462 cultures were stained with the detection molecule FITC-conjugated antibody specific for hFc and then analyzed by flow cytometry. Fig. 2A shows isolation of cell pools expressing Rc1-hFc from RGC10 and RS527.

Three cell bins R4, R5, and R6 marking cells with low, medium, and high fluorescence respectively were sorted from each host line and expanded in tissue culture. To compare Rc1-hFc protein production level from the six cell bins, six cultures were set up using equal number of cells for each bin and conditioned media collected after 3 days. The Rc1-hFc protein titers in the conditioned media were determined by ELISA and were plotted against mean fluorescence of the respective cell bins (Fig. 2B). For both RGC10 and RS527 lines, there was a similar correlation between mean fluorescence (amount of Rc1-hFc displayed on the cell surface) and sPOI protein production levels of the isolated cell pools. The sPOI titers in the two high fluorescence R6 bins derived from RGC10 and RS527 were similar.

5. A second experiment was conducted with a receptor protein as a CSCM. In this example, the CSCM was the hTie2 receptor, and the sPOI was a Tie-specific ScFv-Fc fusion protein (ScFv_{C1b}-Fc).

To construct an inducible Tie2 CSCP cell line, CHO K1 was first stably transfected with the TetR plasmid pcDNA6/TR. The blasticidin-resistant cell pool was then stably transfected with pTE259, a plasmid that allows inducible expression of a protein comprised of the extracellular domain and transmembrane domain of Tie2. Inducible cell clones were isolated by flow cytometry after staining with an antibody specific for Tie2 (Fig. 3).

The RGC54 clone inducibly expressing hTie2 lacking the TK domain was selected for further studies. RGC54 cells were stably transfected with pTE988, a plasmid capable of expressing the secreted hFc-fusion protein ScFv_{C1b}-Fc and conferring resistance to hygromycin. The transfected culture was selected with hygromycin for two weeks. The hygromycin-resistant cells were induced with Dox and blocked with 1 mg/ml of purified C1b mAb. The C1b monoclonal antibody was the source of the variable regions in ScFv_{C1b}-Fc. The next day, the cell pool was stained by a FITC-conjugated antibody specific for hFc (does not recognize the murine Fc of the C1b mAb) and then analyzed by flow cytometry (Fig 4A).

Three cell bins R6, R7, and R8 marking cells with high, medium, and low fluorescence respectively were sorted and expanded in tissue culture. Three cultures were set up using an equal number of cells for each bin to determine ScFv_{C1b}-Fc protein production as determined by ELISA. Fig. 4B shows that a correlation existed between mean fluorescence (amount of ScFv_{C1b}-Fc binding to Tie2 on the cell surface) and ScFv_{C1b}-Fc protein production levels of the isolated cell pools.

6. A third experiment was conducted to investigate the minimum affinity required between the CSCM and the sPOI.

A chimeric protein comprised of angiopoietin-1 receptor binding domain and hFc (FD1-hFc) which binds Tie2 with an affinity constant of 174 nM (as determined by BIAcore) (dissociation $t_{1/2}$ = 1.76 min) was used as the sPOI, and Tie2 was used as the CSCM.

In cell decoration experiments, exogenously added FD1-hFc bound specifically to RGC54 cells through Tie2 (Fig. 5).

RGC54 cells were stably transfected with pTE942, a plasmid capable of expressing FD1-hFc and conferring resistance to hygromycin. The transfected culture was selected with hygromycin for two weeks. The hygromycin-resistant cells were induced with Dox and blocked with 1 mg/ml of purified FD1-mFc comprising mouse IgG1 Fc not recognized by the detection molecule. The next day, the cell pool was stained by a FITC-conjugated antibody specific for hFc and then analyzed by flow cytometry (Fig. 6A).

Three cell bins R6, R7, and R8 marking cells with high, medium, and low fluorescence, respectively, were collected. Cultures were set up using equal number of cells for each bin to determine FD1-hFc protein production levels in the conditioned media as determined by ELISA. Fig. 6B shows that there was a correlation between mean fluorescence (FD1-Fc binding to cell surface-bound Tie2) and FD1-hFc protein production levels of the isolated cell pools. The bin with the highest fluorescence produced the most FD1-hFc.

7. A fourth experiment was conducted to investigate the use of an antibody or ScFv as CSCM. The RS655 cell line, derived from CHO K1, constitutively expresses ScFv_{HB58}-TM_{PDGFR}, a

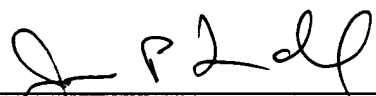
molecule capable of binding murine antibody kappa chain. Cells expressing ScFv_{HB58}-TM_{PDGFR} can be stained by sequential incubation with P12 mAb (specific for FD2), FD2-hFc, and FITC-conjugated anti-hIgG (Fig. 7). P12 captured on the cell surface by the HB58 ScFv was detected by its affinity for FD2, which in turn was detected by recognition of the hFc tag. RS656 cells were derived from RS655 cells after stable transfection with a plasmid encoding the gene for eYFP (Fig. 8A). Nearly 100% of RS656 cells were eYFP-positive, and most (76%) maintained expression of ScFv_{HB58}-TM_{PDGFR} as detected by binding to FD2-hFc (Fig. 8B).

RS655 cells were stably transfected with pTE693, a plasmid capable of expressing the heavy and light chains of the P12 antibody, and conferring resistance to puromycin. The transfected culture was selected with puromycin for two weeks to yield a pool of cells that were heterogeneous with regard to P12 mAb expression (RS655/pTE693).

Equal numbers of RS656 cells and RS655/pTE693 cells were mixed and co-cultured. When P12 expressed from RS655/pTE693 cells was allowed to diffuse and bind to ScFv_{HB58} on the surface of neighboring RS656 cells a large population of yellow cells were also positive for binding FD2-hFc (Fig. 8C). However, if the ScFv_{HB58} on the surface of RS656 was bound with excess murine IgG, then only non-yellow cells were positive for binding FD2-hFc (Fig. 8D) demonstrating that expressing cells were effectively separated from non-expressing cells.

8. I hereby declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and further that I make these statements with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under §1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

11/17/04
Date


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